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HEMICELLULOSE FRACTIONS AND ASSOCIATED PROTEIN OF LUPIN HYPOCOTYL CELL WALLS

JOHN A. MONRO, RAYMOND W. BAILEY,

Applied Biochemistry Division, D.S.I.R. Private Bag, Palmerston North, New Zealand

and

DAVID PENNY

Department of Botany and Zoology, Massey University, Palmerston North, New Zealand

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Abstract—The alkali extraction of polysaccharide fractions from depectinated primary cell walls of lupin hypocotyls was studied using sequential extractions at 0° and 18–22°. Aqueous 10% KOH at 0° removed hemicellulose-A (95%) heteroglycan-B (80%) and linear 1–4 linked hemicellulose-B (60%). Arabinose accounts for 88% of the monosaccharides of the linear 1–4 linked hemicellulose-B extracted between 2 and 5 h at 18–22°. Extraction of the 0° and 18–22° alkali-soluble fractions by denaturants, was also examined. 6M guanidine thiocyanate removed about 60% of the 0° 10% KOH soluble polysaccharide but little of the 18–22° soluble material. Although rapidly extracted by 10% KOH at 0° the hemicellulose-A is not extracted by this reagent. Analyses of cell walls and extracted fractions showed that there is little change in amino acid composition and little extraction of wall protein upon removal of about 60% of total wall hemicellulose with 10% KOH at 0°. It is therefore not bound to the wall through galactosylserine links. 10% KOH at 18–22° caused a marked change in composition and extracted most of the wall protein. An alkali resistant fraction high in hydroxyproline and low in serine was not extracted by 24% KOH at 18–22° in 24 hr.

INTRODUCTION

Polymers in growing plant cell walls bind together by covalent and non-covalent bonds [1]. The degree to which the two types of linkage are responsible for cohesion of total wall polymers is not clear, although polymer cross-linking is central to most hypotheses which explain the growth of primary cell walls. Keegstra *et al.* [2] suggest that the polysaccharide and protein polymers of the primary wall matrix are all glycosidically linked to form a macromolecular complex, while at the other extreme Rees and Wight [3] suggest that intact plant walls can be completely dispersed without breaking covalent bonds.

We have found [4] that some polymer can be removed from depectinated lupin hypocotyl cell walls with urea, sodium dodecyl sulphate (SDS) and guanidinium thiocyanate (GTC), reagents known to disrupt non-covalent but not glycosidic bonds. However, most of the matrix requires more vigorous conditions for its extraction. Treatment with 10% KOH at 0° for 4 hr removes about two thirds of the 10% KOH soluble material (most of the hemicellulose) while the remainder is extracted at 18–22°. The latter extract contains most of the wall hydroxyproline, indicating the presence in it of the proposed wall glycoprotein, extensin. Much of the extensin hydroxyproline [5] and serine [6] are known to be glycosidically linked to sugars, and are postulated to play a role in the control of wall extensibility by covalent cross-linking to the polysaccharides [5]. Release of extensin from the wall under alkaline conditions is explained by the rupture of galactosylserine [6] and peptide bonds. The time course of polymer extraction with 10% KOH at 0° and subsequently at 18–22° has also been examined [7]; hydroxyproline and monosaccharide analyses showed that the composition of the extracted non-dialysable polymer varied with time over the extraction sequence.

Our studies of such time course extractions have now been extended to include the various polysaccharide fractions present in the extracted polymer. The extent to which denaturants (GTC and urea) can extract the same wall polymers which are soluble in 10% KOH at 0° and 18–22° respectively, has also been studied. The fate of extensin during extraction has been further investigated by examining the constituent amino acids of the various fractions isolated from the alkaline extracts. These and other results are discussed in relation to the structure of the primary plant cell wall.

RESULTS AND DISCUSSION

Extraction of polysaccharide fractions from depectinated lupin hypocotyl cell walls

A time course of 10% KOH extraction of polymer from neutral detergent extracted cell walls was conducted as outlined in Table 1. At the end of each stage in the sequence the flask contents were filtered, the filtrate acidified and the residue subjected to the next stage in the sequence. Acidified filtrates were left overnight and then centrifuged to remove the precipitate of hemicellulose-A. The hemicellulose-B supernatants were fractionated

Table 1. Sequence of 10% KOH extractions of depectinated Lupin hypocotyl cell walls

Stage in sequence	1	2	3	4	5	6	7
Filtering time (from sequence start) hr	4	12	14·5	17	19·5	30·5	58·5
Duration of extraction (hr)	4	8	2·5	2·5	2·5	11	28
Temperature of extraction	← 0 →		←	18–22°		→	

into linear 1–4 linked polysaccharide and more highly branched heteroglycan-B by iodine precipitation of the former from $CaCl_2$ solution as described by Gaillard and Bailey [8]. Subsequent work (see below) showed the iodine precipitated polysaccharide to consist of 2 fractions both of which gave, in $CaCl_2$ a heavy blue precipitate with iodine. These were (a) a fraction dispersed but not dissolved in $CaCl_2$ ("$CaCl_2$ insoluble") and (b) a truly soluble fraction. In the first part of this work these fractions were isolated together and are referred to as *total* 1–4 linked linear hemicellulose-B. Amounts of the hemicellulose fractions are shown in Fig. 1. The sum of the fractions extracted per hr indicates that the rate of extraction of total hemicellulose is, at both temperatures, initially rapid but declines, especially at 0°. A striking feature of Fig. 1 is the almost complete extraction of hemicellulose-A (over 95%) during stage 1 of the extraction sequence. Apparently the hemicellulose-A is either bound to the wall by bonds which are extremely alkali labile or is not bonded at all.

About two thirds of the 1–4 linked polysaccharide (iodine precipitated) was extracted at 0°. It therefore showed a distribution between the 0° and 18–22° 10% KOH fractions similar to that previously found [4,7] for the total polymer of lupin hypocotyl cell walls. Most of the heteroglycan-B was also extracted by 10% KOH at 0°, although apparently at a slower rate than other fractions. A slight increase occurred during stages 4 and 5 of the extraction sequence at which points much of the polymer was possibly derived from degradation of the extensin–polysaccharide complex of the wall [7].

Composition of polysaccharide in fractions isolated from 0° and 18–22° 10% KOH extracts

Monosaccharide compositions of the fractions shown in Fig. 1 are presented in histogram form in Fig. 2. In *hemicellulose-A* the predominant monosaccharide was xylose (74·2%) and although it was only 12% of the polymer extracted during stage 1 of the sequence it would contribute significantly to the high xylose level previously reported [7] for total polymer extracted during the first hour at 0°. There is a sharp increase in glucose content from 6 to 60% of the hemicellulose-A between stages 1 and 4 of the extraction sequence. The hemicellulose-A is however a very minor component of polymer extracted after stage 1.

Total 1–4 *linked hemicellulose-B*, being the bulk of the extracted polymer, showed the same trends in monosaccharide composition over the sequence as were found in total polymer [7]. The overall composition of the lupin hypocotyl total 1–4 linked hemicellulose-B, in containing significant amounts of galactose, is different to

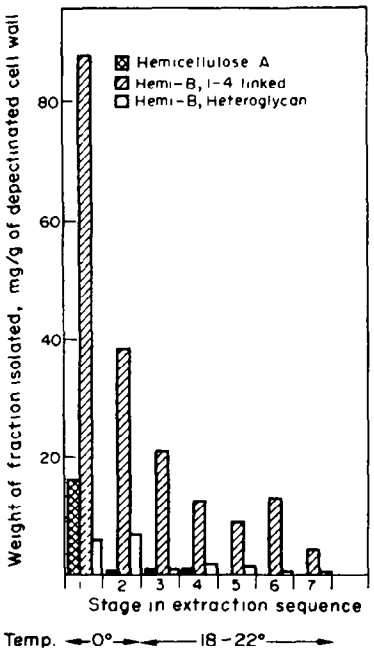

Fig. 1. Fractions isolated from extracts of sequence shown in Table 1.

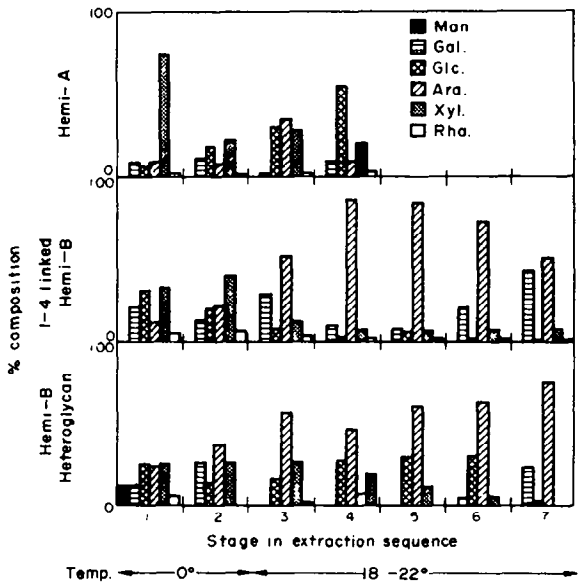

Fig. 2. Monosaccharide composition of fractions isolated from extracts of sequence shown in Table 1.

that reported [9] from a number of legumes and grasses. This could be because lupin hypocotyl contains mainly primary wall whereas a high degree of secondary thickening had occurred in the legumes and grasses [9]. The high galactose level of the hypocotyl fraction might also reflect more extensive cross-linking of wall polymers through galactosylserine bonds to extensin.

The 0° and 18–22° hemicellulose-B fractions were of different composition. Thus material extracted at 0° contained between 20 and 40% each of xylose and glucose. In contrast, as a percentage of total 1–4 linked hemicellulose-B from each stage in the 18–22° extraction there was (a) a decrease in galactose followed by a rise and (b) an increase in arabinose to 86% of 1–4 linked polymer at stage 4 followed by a slight decline. This pattern probably reflects the release of peptide bound arabinose on fragments of extensin, which has a high level of hydroxyproline substituted with (arabinose) 1–4 linked oligosaccharides [5]. The maximum rates of arabinose and hydroxyproline release by 10% KOH at 18–22° after prior 0° 10% KOH extraction have already been shown to coincide [7]. Furthermore, differential dialysis of arabinose and galactose-containing material from alkaline hydrolysates of total wall suggests that the arabinose resides partly in fragments which are smaller than those containing galactose [10].

Hemicellulose-B heteroglycan was at all stages a minor fraction and most was extracted at 0°, during stages 1 and 2. Its composition at these stages was similar to that already reported for such heteroglycan from legumes and grasses [9] with the exception of the presence of mannose, which is typical of polysaccharide tightly associated with the cellulose microfibrils. Such mannose polymers generally require treatment with 24% KOH at room temperature to extract them [7,8] and even then some mannose usually remains in the 24% KOH-insoluble "cellulose" fraction. The mannose polymer in the heteroglycan-B differs from that in the 24% OH soluble hemicellulose both in its facile extraction and in its nonprecipitation from $CaCl_2$ solution by iodine.

Heteroglycan-B extracted at 18–22° differs from that extracted at 0° and from total hereroglycan-B of other plants in that it contains virtually no galactose and has higher levels of arabinose. The arabinose levels are consistent with the presence of peptide fragments bearing arabinose oligosaccharide side chains. Hydroxyproline has been shown to comprise about 3% of the heteroglycan-B from total hemicellulose but to be absent from the 0° soluble polysaccharide [7]. The total heteroglycan-B hydroxyproline therefore resides solely in the 18–22° heteroglycan-B which would be about 12% hydroxyproline by weight. Most of the arabinose could therefore be accounted for as hydroxyproline-linked tetraarabinosides.

Centrifugation of total 1–4 linked hemicellulose-B in solution in $CaCl_2$ S.G. 1·3

When the $CaCl_2$ solution of hemicellulose-B, clarified by brief centrifuging, is subject to prolonged centrifuging before adding iodine much polymer is spun down as a gelatinous mass. The monosaccharide composition and weights of total 0° and 18–22° hemicellulose-B separated into material spun from $CaCl_2$ solution and present in the supernatant, respectively, are shown in Table 2. It is evident that some degree of separation has been achieved with the "$CaCl_2$ insoluble" fractions enriched in galactose and arabinose. When the "$CaCl_2$ insoluble" material was resuspended in $CaCl_2$ solution and I_2KI added a dense blue precipitate was formed indicating the presence of predominantly linear 1–4 linked polysaccharide [11]. The presence of 7M urea in the $CaCl_2$ solution prevented the "$CaCl_2$ insoluble" material from being spun down, suggesting that the insolubility was at least partly due to polymer interactions in solution. The extent to which the "$CaCl_2$ insoluble" polymer contained material not precipitated by iodine was not investigated. Whether the "$CaCl_2$ insoluble" galactose–arabinose polymer is an extensin–polysaccharide complex or a linear arabinogalactan which can react with iodine in $CaCl_2$ remains to be established. Iodine precipitation in $CaCl_2$ requires the polymer to have a helical configuration [11]. It is doubtful that peptide linked tetraarabinose could form a helix.

$CaCl_2$-insoluble and $CaCl_2$-soluble hemicellulose-B from high and low growth rate regions of Lupin hypocotyl

It has been shown that the total 1–4 linked polymer from the lower 2 cm (non growing [10]) region of a 6 cm lupin hypocotyl has a greater proportion of arabinose and galactose relative to xylose than the upper rapidly growing 2 cm region [4]. This is consistent with the greater content of extensin in the lower region, where it could cross link via arabinosylhydroxyproline and galactosylserine to other wall polymers. To see whether arabinose and galactose increases could be localized in either the $CaCl_2$ insoluble or $CaCl_2$ soluble iodine precipitable fractions of Table 2 these fractions were separated in hemicellulose extracts from walls of both lower and upper hypocotyl regions. The results are shown in Table 3. Although increases in arabinose between upper and lower regions occur in both fractions an increase in galactose is seen only in the $CaCl_2$ soluble 1–4 linked fraction.

Amino acid composition of polysaccharide fractions of lupin hypocotyl

Additional information on the involvement of cell wall protein in the polysaccharide fractions was obtained

Table 2. Extraction of $CaCl_2$-soluble and $CaCl_2$-insoluble hemicellulose from 6 cm Lupin hypocotyl cell walls by 10% KOH

Extraction temperature	Fraction	Weight*	Monosaccharide composition (relative to xylose) Xyl.	Ara	Glc	Gal
0	$CaCl_2$-soluble	86	1	0·19	0·38	0·48
0	$CaCl_2$-insoluble	47	1	1·01	0·36	1·65
20	$CaCl_2$-soluble	18	1	1·45	0·29	0·66
20	$CaCl_2$-insoluble	24	1	3·48	0·28	4·74

* mg/g of neutral detergent extracted cell wall.

Table 3. Extraction of $CaCl_2$-soluble and $CaCl_2$-insoluble hemicellulose from upper and lower 2cm regions of 6cm Lupin hypocotyls by 10% KOH

Hypocotyl regions	Fraction*	Weight†	Monosaccharide composition (Relative to Xylose) Xyl	Ara	Glc	Gal
Upper (growing)	$CaCl_2$-soluble	23	1	0·59	0·41	0·61
	$CaCl_2$-insoluble	35	1	3·19	0·24	5·31
Lower (non growing)	$CaCl_2$-soluble	28	1	1·90	0·43	1·07
	$CaCl_2$-insoluble	31	1	4·49	0·16	4·96

* Extraction for 24 hr at 20° after prior extraction for 10 hr at 0°. † mg/g of neutral detergent extracted cell wall.

from the amino acid composition of the fractions. A variety of amino acid-carbohydrate bonds are possible [5] and enrichment of a particular amino acid in any fraction might indicate a bond between this amino acid and a polysaccharide specific to the fraction.

Hypocotyl cell walls were fractionated as before. Amounts of fractions, extracted walls, and protein contents calculated from amino acid analyses are shown in Table 4. Amino acid compositions of the fractions and residues are shown in Table 5. The 0° soluble polymer was not analysed as it has already been shown to contain little extensin [7].

Figures for total protein/mg of sample shown in Table 4 confirm that little protein is removed with 10% KOH at 0° and most of it (77%) at 18–22°. The 24% KOH at 18–22° removed *ca* 55% of the protein not extracted with 10% KOH, leaving *ca* 10% of the protein in the original depectinated walls.

Extraction with 10% KOH at 0° caused little change in amino acid composition of the depectinated walls (Table 4). Release of polysaccharide by 10% KOH at 0° does not therefore require β-elimination and consequent destruction of serine. This is contrary to the wall model of Keegstra *et al.* [2] which predicts that xylose-containing polymer should not be extracted unless accompanied by β-elimination of galactosylserine and simultaneous extraction of protein. The 0° 10% KOH soluble hemicellulose must be bound into the wall by other more alkali labile links which might still involve extensin.

Extensin from a wide variety of plants has proved to be remarkably uniform in composition, especially with regard to hydroxyproline levels (*ca* 30% in most cases [5,14]), and confined almost totally to the cell wall. The values reported here are similar to those already published and attest to the efficiency of the neutral detergent in clearing the walls of cytoplasmic protein.

Extraction of the 0° 10% KOH residue at 18–22° for 24 hr causes a marked change in amino acid composition of the residual wall. As a percentage of amino acids in the residue hydroxyproline and serine drop to about 75 and 50% respectively of their values for protein of the depectinated walls. The drop in serine probably indicates that β-elimination of galactosylserine has occurred, although serine is inherently unstable at high pH. The increase in glycine values in the 18–22° 10 and 24% extracted residues also probably reflects this.

Compositions of proteins in the various fractions extracted by 10% KOH at 18–22° show considerable variation. Uncertainties due to dialysis losses and alkaline degradation make it difficult to obtain a basis for comparison of the various fractions. The linear 1–4 hemicel-

Table 4. Extraction of polysaccharide and protein from depectinated Lupin hypocotyl cell walls

Extractant*	Wall fraction	Weight of fraction mg/g depectinated wall	Protein mg/g sample	Protein mg/g depectinated wall	% Extraction of depectinated cell wall protein
1. Neutral detergent–EDTA 100°, 3 hr	Depectinated cell wall	1000	24·3	24·3	
2. 10% KOH, 0°, 8 hr	Total 0° hemicellulose	103 (288)†	—	—	
	Wall	765	33·0	25·2	0
3. 10% KOH 18–22° 24 hr	Total 18–22° hemicellulose	45·3 (85·4)†	—	—	
	linear 1–4 $CaCl_2$ insoluble	23·0	133	3·05	
	heteroglycan-B	10·6	156	1·66	
	Wall	4·1	163	0·67	
		627	8·9	5·63	77
4. 24% KOH 18–22° 24 hr	Total 24% KOH hemicellulose	30·0	—	—	
	Wall	512	5·02	2·57	89·4

* Extractions in sequence. † Figures in parentheses weights after 24 hr dialysis, others after 48 hr.

Table 5. Amino acids of the fractions and residues shown in Table 4 as a percentage of total amino acids for each analysis

Amino acid	Sample and amino acid content (%) Extracted walls: Neutral detergent (a)	10% KOH: 0° (b)	10% KOH: 18–22° (c)	24% KOH (d)	10% KOH, 18–22° polysaccharide fraction: Linear 1–4 (e)	$CaCl_2$ insoluble (f)	Heteroglycan-B (g)
Lysine	9·40	9·59	6·23	5·53	9·85	9·34	6·40
Histidine	4·95	5·95	2·22	1·58	6·63	8·51	1·57
Arginine	0.99	0·97	1·33	0·81	0·54	1·69	3·61
Hydroxyproline	31·08	28·31	21·02	29·58	36·31	20·76	9·46
Aspartic acid	3·67	3·13	5·45	4·40	2·81	4·14	8·03
Threonine	2·51	2·09	2·22	1·65	1·80	2·35	3·72
Serine	10·34	10·65	4·67	2·27	9·74	6·64	13·57
Glutamic acid	3·42	3·19	4·67	4·17	3·49	6·12	15·14
Proline	8·70	8·56	9·57	10·13	8·69	12·47	2·48
Glycine	7·25	6·49	14·79	12·61	1·12	3·68	8·88
Alanine	2·72	2·52	3·00	3·07	2·27	2·87	3·74
Half-cystine	—	—	—	—	—	—	—
Valine	4·78	4·79	5·34	5·07	4·61	5·59	3·55
Methionine	0·25	0·24	0·33	0·26	0·18	0·45	1·11
Isoleucine	2·23	2·21	4·12	4·57	1·43	2·25	3·07
Leucine	3·46	3·35	5·12	5·04	2·43	4·91	5·38
Tyrosine	5·11	4·79	3·23	1·99	6·50	4·50	4·41
Phenylalanine	2·72	2·67	6·17	6·48	0·87	2·01	2·79

lulose-B protein (Table 5) has a similar amino acid composition to that of the depectinated wall. This suggests that the linear 1–4 hemicellulose-B protein is that which has not yet been β-eliminated from association with polysaccharide. The heteroglycan-B fraction also has relatively high serine levels. This latter polymer is however, a minor fraction which contains only a small proportion of the neutral detergent extracted wall protein.

Hydroxyproline levels vary between residues and fractions. In its glycosylated and non-glycosylated states it is stable to both acid and alkali and is therefore not likely to have been destroyed. Lack of enrichment of hydroxyproline in the residue suggests that its arabinosyl side chains are not further attached to microfibril-linked polysaccharide, contrary to the wall model of Lamport [5].

The variation in hydroxyproline levels suggests that its distribution along the peptide chain is uneven. In the $CaCl_2$ soluble linear 1–4 hemicellulose-B fraction there are high levels of hydroxyproline and serine compared with either the 10% KOH 18–22° extracted wall or the $CaCl_2$ insoluble material. This is possibly explained by proximity of hydroxyproline and serine in the extensin peptide chain. In the isolated fragments of extensin so far sequenced the lowest hydrocyproline content was 60% of constituent amino acids [6,15] and the fragment in which galactosylserine was identified [6] had the composition $Galactose_2$ $Serine_2$ $Hydroxyproline_5$ $Lysine_1$. In most cases hydroxyproline and serine predominate so that it appears that where galactosylserine links exist, there is an enrichment of the polymer in hydroxyproline. The linear 1–4 hemicellulose-B composition is consistent with this, although galactosylserine links have not been demonstrated. It is however, relevant that a high level of hydroxyproline is present in a fraction with high serine levels and in which the protein and polysaccharide have shown parallel solubility in $CaCl_2$ S.G. 1·3 and precipitability with I_2KI.

The 24% KOH extracted walls have a very low serine level and compared with the 18–22° 10% KOH extracted walls are high in hydroxyproline. The lack of enrichment in serine and its destruction during β-elimination of galactosylserine, known to occur under the conditions used [6], suggests that the protein is not bound into the 24% KOH extracted walls by galactosylserine, as suggested in the wall model of Keegstra *et al.* [2]. Alkali stable protein has been found in a variety of plants [14,15]. The nature of this tenaciously bound protein is of interest in view of the suggestions [2,5] that the extensin-polysaccharide complex of the wall matrix controls wall extensibility by crosslinking between the cellulose microfibrils. Whether or not covalent bonding is involved, the alkali resistant wall fraction deserves further attention.

Extraction of 0° *and* 18–22° 10% *KOH soluble polymer fractions from depectinated cell walls by Urea and GTC*

To assess the degree to which breaking non-convalent bonds results in the release of the 0° and 18–22° 10% KOH-soluble polymers depectinated (neutral detergent–EDTA, 3 hr) cell wall was extracted with either urea or GTC for 18 hr prior to the sequential extraction in 10% KOH at the above temperatures. The results are shown in Table 6. GTC is a powerful chaotropic agent [16] and is clearly more effective than urea in removing polymer. It does so almost entirely at the expense of the 0° 10% KOH soluble fraction to the extent of 60% of that fraction.

The present work suggests that about 40% of the lupin hypocotyl hemicellulose is bound into the wall by non-covalent bonds. Although a time course of GTC extraction has not been made, it would seem that since it was not extracted after 16 hr, the 0° 10% KOH soluble fraction not extracted by GTC is indeed bound into the wall by other than hydrophobic or hydrogen bonds. The extent of extraction of polymer during the initial stages of the 0° extraction indicates that very alkali labile

Table 6. Urea and guanidine thiocyanate (GTC) pre-extraction of polymer released from depectinated cell wall by 10% KOH

Wall fraction or extractant*	Polymer released (mg/g of depectinated cell wall) (a)†	(b)	(c)
8M Urea, 16 hr	– ‡	51·6	--
6M GTC, 16 hr	143·3	---	---
10% KOH, 0°C, 6 hr	91·6	144·9	198·3
10% KOH, 18–22°, 20 hr	90·6	96·9	98·9
Wall residue	681·6	675·3	677·9

* Extractions are in sequence. † (a), (b), and (c) differ in the pre-10% KOH treatment, as shown.
‡ Indicates extraction step omitted.

bonds, such as ionic bonds, are being broken. These are unlikely to involve Ca^{2+} stabilization [17] of polyuronide association as the EDTA in the neutral detergent-EDTA used to extract the walls prior to the alkali treatment effectively chelates Ca^{2+} and for this reason is highly effective in removing pectins. That the GTC soluble material does not contain pectin is also suggested by the ineffectiveness of GTC in removing polyuronide from mung bean hypocotyl [18] or from lupin hypocotyl (unpublished).

Monosaccharide compositions of the fractions in Table 6 are given in Table 7. The GTC soluble polymer differs from the 0° 10% KOH polymer in containing most of the wall rhamnose. Some fractionation therefore appeared to occur. This was confirmed when a comparison of hemicellulose-A levels from depectinated walls extracted with 6M GTC, 8M urea and 8M urea–$CaCl_2$ respectively were compared. None of the denaturants removed significant quantities of hemicellulose-A. The hemicellulose-A, therefore, does not depend solely on hydrogen or hydrophobic bonds for its retention in the wall but, as suggested by its rapid release by 10% KOH at 0°, possible relies on weak ester linkages involving uronic acids. The precipitation of hemicellulose-A which occurs when alkaline wall extracts are acidified does in fact depend on the protonation of carboxylic acid groups so that aggregation of the polysaccharide chains can occur [17]. The inability of pectinase to remove more than a small proportion of polymer from the depectinated walls (unpublished) suggests that the uronic acids are involved as side chains rather than as xylan-linked polyuronide.

Results of the sequential extractions reported here suggest the following bonds in lupin hypocotyl cell walls. (1) Ca^{2+} stabilized bonds involving uronic acids. When these are broken by treatment with hot detergent–EDTA or oxalate the pectic fraction is released. (27% of the total cell wall). (2) Hydrogen or hydrophobic bonds broken by 6M GTC to release at least 8% of the wall. (3) Bonds very labile to 10% KOH at 0° (but not to 6M GTC) and which when broken release the xylose rich hemicellulose-A (95% removal of hemicellulose-A in the first 4 hr of extraction). The hemicellulose-A is at least 1·8% of the total cell wall. (4) Bonds labile to 10% KOH at 0° but apparently less so than those involving hemicellulose-A. and stable in 6M GTC. These probably involve at least 4% of the total wall. (5) Bonds broken in 10% KOH at 18–22°. These include some peptide bonds and galactosylserine linkages and their rupture releases at least 5% of the total wall including most of the extensin. (6) Bonds not broken after 24 hr in 10% KOH at 18–22° but which are labile to 24% KOH at 18–22°. The 24% KOH releases at least 3% more wall after 24 hr. (7) Bonds stable in 24% KOH under the conditions of (6). These appear to involve both protein and hemicellulose.

While a large proportion of the lupin hypocotyl primary cell wall polymers are clearly not covalently bound into the total polysaccharide–glycoprotein complex a substantial proportion of the wall is resistant to the action of agents usually effective in breaking non-covalent bonds. If cuprammonium solutions are capable of dispersing the whole undelignified cell wall it is likely that their effectiveness is due partly to their alkalinity causing degradation of a polysaccharide–glycoprotein complex.

It is apparent that a number of different covalent and non-covalent linkages are involved in the binding together of plant cell wall matrix polymers. Their sequential disruption achieved in the experiments reported here shows that different polymers are involved in greater variety of linkages and in a manner different than suggested in a recent model of the primary cell wall [2], where the whole was united to form a glycosidically linked macromolecule.

EXPERIMENTAL

Plant tissue. Lupin hypocotyls, 6 cm (entire unless otherwise stated) were grown and prepared as previously described [10].

Cell wall preparation. Hypocotyls (300 g) were ground for 30 sec in dist. H_2O in a Waring blender. filtered through sintered glass and immediately extracted under reflux for 3 hr in 2 changes of 1·5 l. of neutral detergent–EDTA of Van Soest [19]. The neutral detergent removes cytoplasmic material and non-covalently bound protein and pectins from the wall. The

Table 7. Monosaccharide composition of polymers of Table 6, extraction sequence (a)

Extraction	Monosaccharide (mg/g of depectinated cell wall) Xyl	Ara	Man	Glc	Gal	Rha
Guanidine thiocyanate	31·8	21·5	3·2	39·4	15·2	32·2
10% KOH, 0°	61·8	15·1	0·8	2·4	10·4	1·2
10% KOH, 18–22°	7·2	67·9	0	3·4	9·4	2·6

walls were filtered, washed 2× by resuspending in hot dist. H_2O and filtering, and finally washed with EtOH and then Me_2CO and dried at 40° for 18 hr. This gave depectinated cell wall.

Extractions. Alkali extractions involved stirring on a magnetic stirrer in a sealed flask under N_2 for the specified time and temp, using about 1 g tissue/100 ml alkali. For the time course, depectinated wall was subjected to a series of extractions. The number of each extraction in the sequence, the time of filtering of each extraction (taken from the start of stage 1 in the sequence) and the temperature and duration of each stage are shown in Table 1. At the end of each stage the walls were filtered and then immediately submitted to the next extraction in the sequence. Except for non-dialysed samples for amino acid analysis extracts were adjusted to pH 4·5 with HOAc immediately after filtering and dialysed for 48 hr before freeze drying. Non-dialysed samples for amino acid analysis were neutralized with HCl and evaporated to dryness in a rotary evaporator at 40°.

8M urea and 6M GTC extractions involved stirring with a magnetic stirrer in a closed flask overnight at room temp. using 0·5 g cell walls to 100 ml sol. The cells were filtered and washed 2× by resuspending in dist. H_2O and filtering. Filtrate and washings were combined and dialysed for 48 hr.

Polysaccharide fractionations. Separation of hemicellulose-A and hemicellulose-B was achieved by centrifuging the acidified alkali extracts at 70000*g* for 1 h after standing at 0° overnight. The ppt. (hemicellulose-A) was resuspended in H_2O and freeze-dried. The hemicellulose-B supernatant (or total polymer where hemicellulose-A not isolated) was dialysed and freeze dried. The freeze dried hemicellulose-B was suspended in $CaCl_2$ S.G. 1·3 by stirring for 18 hr and the soln clarified by centrifugation at 70000*g* for 5 min. Where the $CaCl_2$-insoluble polymer was to be isolated the soln was then centrifuged at 70000*g* for 1 hr. The supernatant in each case was fractionated into linear 1–4 linked hemicellulose and heteroglycan-B by precipitation with I_2 as described in ref. [8].

Carbohydrate analyses. 20 mg samples were hydrolysed with 72% (w/w) H_2SO_4 (0·7 ml, 3 hr, room temp.) made up to 20 ml with dist. H_2O and refluxed for 3 hr. Hydrolysates were neutralized with $BaCO_3$, filtered and freeze dried. Monosaccharides in the hydrolysate were analysed by GLC of their alditol acetates [20] using a 2 m × 2 mm stainless steel column packed with 3% ECNSS-M on *Gas-Chrom* Q.

Amino acid analyses were carried out by column chromatography in an amino acid analyser on samples hydrolysed for 16 hr in 6M HCl.

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